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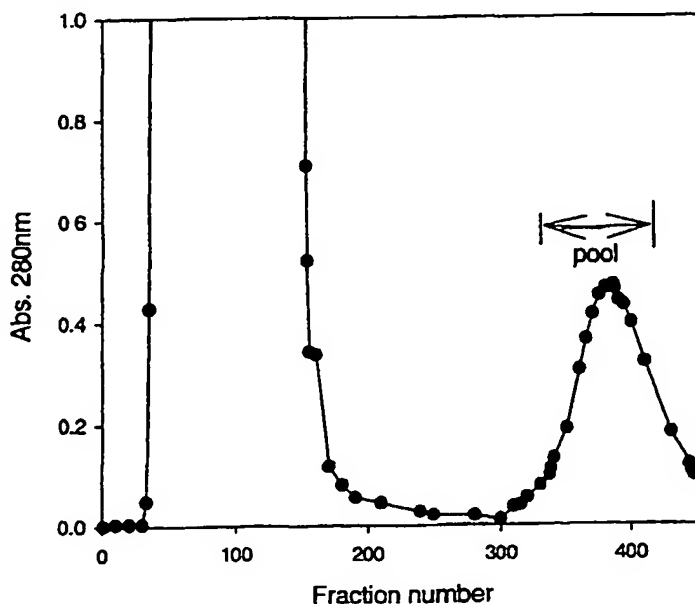
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(54) Title: METHOD FOR MEASURING SERUM ASIALO-GLYCOPROTEIN CONCENTRATION FOR DIAGNOSIS OF HE-
PATIC DISEASE AND KIT THEREFORIsolation of α 1- acid glycoprotein from human plasma
by DEAE-cellulose column chromatography

(57) Abstract: The present invention relates to a method for measuring asialo-glycoprotein concentration through a sandwich assay using lectin as at least one of capture protein and probe protein and a kit therefor. The method and kit of the present invention can be used effectively in early diagnosis and judgement on treatment result of hepatic diseases including liver cirrhosis and hepatocellular carcinoma, said method being able to measure many samples simultaneously as well as are high in safety and reproducibility.



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METHOD FOR MEASURING SERUM ASIALO-GLYCOPROTEIN CONCENTRATION FOR DIAGNOSIS OF HEPATIC DISEASE AND KIT THEREFOR

5 BACKGROUND OF THE INVENTION

The present invention relates to a method for measuring asialo-glycoprotein concentration through a sandwich assay and a kit therefor, more particularly to a method and a kit for measuring the amount of asialo-glycoprotein(ASGP) such as asialo α 1-acid glycoprotein(AS-AGP), asialo haptoglobin(AS-HG), or asialo α 2-macroglobulin(AS-MG) suspected of being increased in the blood in case of an attack
10 of a hepatic disease by using an antibody against glycoprotein and a lectin recognizing asialo-glycoprotein, or using lectins.

Hepatic diseases such as hepatitis, liver cirrhosis, hepatocellular carcinoma are the ones that have the largest number of patients as a single disease in Korea, Japan, Taiwan,
15 China, and the most part of Southeast Asian Countries, and presently, a hepatic disease is diagnosed through the methods such as of checking the level of bilirubin or urobilinogen in urea, of measuring the total amount of GOT(glutamic-oxaloacetic transaminase), GPT(glutamic pyruvic transaminase), bilirubin, albumin, protein, lactic acid dehydrogenase, and the like to observe variation of biochemical components, or of
20 detecting an antigen of hepatitis B virus(HBV) or hepatitis C virus(HCV) or an antibody against them. In addition, hepatocellular carcinoma may be diagnosed through alpha-feto protein(AFP) and carcinoembryonic antigen(CEA) examination. However, treatment of a hepatic disease has difficulty in making a diagnosis in many cases after the liver grew seriously worse due to a technical imperfection of early diagnosis,
25 because the liver performs various complicated functions and has a physiological

feature of not being detected of its abnormality easily. In the case of a hepatic disease developed from acute hepatitis into chronic hepatitis, liver cirrhosis and a tumor of the liver, or from hepatitis into hepatocellular carcinoma, it is necessary to establish an effective diagnostic system for tracing and/or diagnosing their processes, and is also
5 necessary to make an early diagnosis for treating these diseases effectively and preventing the disease from developing into fatal liver cirrhosis or hepatocellular carcinoma. Therefore, a method for diagnosis that can keenly and specifically analyze the marker of a hepatic disease which reflects the development of the hepatic disease in the patient accurately is required.

10 A study on asialo-glycoprotein and asialo-glycoprotein receptor had begun in the early 1970's mainly for animal models and has been in progress from the early 1990's for the human bodies. It has been reported that asialo-glycoprotein is a marker in the serum for reflecting the progressive state of a hepatic disease because it is increasing rapidly in a development stage of an acute hepatitis and decreasing in a convalescent
15 stage (T. Sawamura, et al., Gastroenterology, 1981; 81:527~533). Also, it has been reported that when a hepatitis is induced in an animal, the concentration of asialo-glycoprotein in blood and that of asialo-glycoprotein receptor in liver tissue show a symmetrical aspect each other, and therefore the concentrations thereof are considered as a clinical marker to determine a progressive state of the hepatic disease (T. Sawamura,
20 et al., Gastroenterology, 1984; 87:1217~1221). Further, it has been found that in the case of the hepatocellular carcinoma, the concentration of asialo-glycoprotein in blood and the size of the hepatocellular carcinoma show a direct proportional aspect, and therefore asialo-glycoprotein is a marker indicating the stage of the hepatocellular carcinoma (T. Sawamura, et al., Gastrologia Japonica, 1985; 20:201~208). It has been
25 reported, as described above, asialo-glycoprotein and its receptor have relationship

specific with the function of the liver, and the concentration in blood and that in the liver tissue reflect the function state of the liver or the clinical state of a patient.

Presently, the marker of hepatocellular carcinoma such as AFP and CEA has difficulty in judging the curative effect of a patient because of its low specificity.

5 However, because asialo-glycoprotein and its receptor has high specificity for a hepatic disease, they are expected as an effective marker for an early diagnosis and a judgment on the curative effect of a patient. An attempt to use a substance directly related to the functions of a liver as a marker instead of the existing marker for diagnosis of a hepatic disease will not only present a new direction for a diagnostic technique of a hepatic
10 disease but give a way for an early treatment of the disease by an early diagnosis.

In a prior art, to measure the concentration of asialo-glycoprotein, asialo-glycoprotein receptor is isolated from a human bodies or other animals such as rabbit and rat, purified, and used as a capture protein, and a competitive radioreceptor assay using radioactive labeling substance or an eletroimmunodiffusion is employed (J. S.
15 Marshall, et al., J. Lab. Clin. Med., 1978; 92:30~37 and N. Serbource-Goguel, et al., Hepatology, 1983; 3:356~359). However, the asialo-glycoprotein receptor is difficult to obtain as large an amount as necessary for the development of the kit for measuring asialo-glycoprotein. Further, the competitive radioreceptor assay has many difficulties such as a risk of suffering from radioactive rays and necessity of a particular facility for
20 radioactive waste disposal, due to the use of radioactive substance and the eletroimmunodiffusion has a problem in difficulty of quantitative analysis. Particularly, in the competitive assay, accuracy and reproducibility are so low that the assay is not suitable for the diagnostic kit. To solve above described problems in the prior art, the present inventors have developed a method and a kit for measuring serum asialo-
25 glycoprotein concentration reproducibly and accurately to diagnose liver functions and

treat hepatic diseases.

Among a variety of methods for analyzing a particular substance qualitatively and quantitatively, particle agglutination assay, a radioimmunoassay(RIA), an enzyme immunoassay(EIA) and a fluoroimmunoassay(FIA) are mostly well known. Of these
5 assays, the radioimmunoassay has high sensibility but has many problems due to the use of radioactive labeling substance, so the enzyme immunoassay and the fluoroimmunoassay are more widely used because they are safe and simple as well as high in sensibility. In the enzyme immunoassay, stable enzymes such as alkaline phosphatase, horseradish peroxidase, or glucose oxidase are used generally, and color
10 developer of high turn-over rate is selected and used as a substrate of enzyme. This assay has similar sensibility and specificity to the radioimmunoassay and can solve the problems of the radioimmunoassay, thus bringing on a wide use.

The present inventors have developed a sandwich assay method and a kit for measuring the serum concentration of asialo-glycoprotein effectively by using an
15 antibody against glycoprotein(α 1-acid glycoprotein, haptoglobin, or α 2-macroglobulin) and a lectin recognizing asialo-glycoprotein, said method being able to measure many samples simultaneously as well as are higher in safety and reproducibility than that of the prior art for measuring the concentration of asialo-glycoprotein.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a sandwich assay method for measuring reproducibly and accurately the serum concentration of asialo-glycoprotein suspected of being increased in blood in case of an attack of a hepatic disease.

25 Another object of the present invention is to provide a kit for measuring the asialo-

glycoprotein concentration in samples.

The present invention provides a method and a kit for measuring the concentration of asialo-glycoprotein by using lectin recognizing asialo-glycoprotein as at least one of a capture protein or a probe protein through a sandwich assay to measure the
5 concentration of asialo-glycoprotein being present excessively in the blood when developing from normal into hepatitis, liver cirrhosis, or hepatocellular carcinoma.

More particularly, the method of the present invention comprises: (a) adsorbing an antibody against glycoprotein onto a solid phase such as a microtiter plate, (b) adding serum sample to the solid phase to bind an asialo-glycoprotein in serum to the antibody,
10 (c) adding a lectin coupled with labeling substance to bind it to the asialo-glycoprotein bound with antibody, and (d) detecting the labeling substance to measure the concentration of asialo-glycoprotein.

Further, the method of the present invention comprises: (a) adsorbing a lectin recognizing asialo-glycoprotein onto a solid phase such as a microtiter plate, (b) adding
15 serum sample to the solid phase to bind an asialo-glycoprotein in serum to the lectin, (c) adding a lectin coupled with labeling substance to bind it to the asialo-glycoprotein bound with lectin, and (d) detecting the labeling substance to measure the concentration of asialo-glycoprotein.

Furthermore, the method of the present invention comprises: (a) adsorbing a
20 lectin recognizing asialo-glycoprotein onto a solid phase such as a microtiter plate, (b) adding serum sample to the solid phase to bind an asialo-glycoprotein in serum to the lectin, (c) adding an antibody against glycoprotein coupled with labeling substance to bind it to the asialo-glycoprotein bound with lectin, and (d) detecting the labeling substance to measure the concentration of asialo-glycoprotein.

25 In the present invention, an asialo-glycoprotein may be desialylated a 1-acid

glycoprotein(AGP), desialylated haptoglobin(HG), or desialylated a 2-macroglobulin(MG).

Also, a lectin recognizing specifically asialo-glycoprotein may be *Arachius hypogaea* agglutinin(PNA), *Ricinus communis* agglutinin(RCA), *Agarius bisporus* agglutinin(ABA), or *Viscum album* agglutinin(VAA), and it is more preferable to use
5 PNA or RCA, and most preferable to use RCA.

A labeling substance to be coupled with lectin or antibody may be enzyme or fluorescent material. In the case of using enzyme as a labeling substance, it is preferable to use the enzyme of high stability such as alkaline phosphatase, horseradish peroxidase,
10 or glucose oxidase, and the amount of asialo-glycoprotein can be measured by adding a color developer of high turn-over rate, for example ortho-phenylene diamine(OPD). In the case of using fluorescent material as a labeling substance, it is preferable to use fluorescein, or rhodamine, and the amount of asialo-glycoprotein can be measured by analyzing fluorescent strength.

15 More specifically, the method of the present invention , for example, comprises as follows: adding an antibody against glycoprotein to a solid phase such as a microtiter plate well, allowing it to stand more than 1 hour to adsorb the antibody onto the microtiter plate well, adding a bovine serum albumin solution to adsorb the albumin onto remaining spaces of well, washing the well with a detergent such as a phosphate
20 buffer containing a Tween-type surfactant, adding a dilution of the serum sample to each well to react them at room temperature, washing the well with above detergent again to react them at room temperature, adding lectin labeled with an enzyme or fluorescent material such as a RCA-horseradish peroxidase complex to each well to react them at room temperature, washing the well with above detergent, adding color-
25 developer of enzyme such as ortho-phenylene diamine solution, stopping the reaction

after a predetermined period, measuring absorbance at an appropriate wave length, and determining the concentration of serum asialo-glycoprotein by comparing the obtained absorbance with that of the standard solution for asialo-glycoprotein. If the labeling substance is a fluorescent material, fluorescent strength is measured after lectin labeled with the fluorescent material is added to each well, reacted them at room temperature and washed them with a detergent.

Alternately, the present invention includes a modified method that replacing above antibody against glycoprotein with lectin recognizing asialo-glycoprotein, the lectin is adsorbed onto a microtiter plate well and the antibody against glycoprotein labeled with a labeling substance is used, and a modified method that the lectin is adsorbed onto a microtiter plate well and the lectin labeled with a labeling substance is used.

According to the method of present invention, if asialo α 1-acid glycoprotein is used as the reference material, the serum asialo-glycoprotein can be detected at the range of $0.03\mu\text{ g/ml}$ ~ $4\mu\text{ g/ml}$.

Also, the present invention provides a kit for measuring the level of asialo-glycoprotein that comprises a solid phase adsorbed with an antibody against glycoprotein or a lectin and a lectin coupled with labeling substance. Preferably, the kit of the present invention comprises a solid phase adsorbed with an antibody against α 1-acid glycoprotein, haptoglobin or α 2-macroglobulin, or a lectin recognizing asialo-glycoprotein, a lectin solution coupled with labeling substance, a detecting solution of labeling substance, a serum dilution, detergent, and a standard solution for asialo-glycoprotein. If the labeling substance is an enzyme, a substrate solution of enzyme is used as the detecting solution. If the labeling substance is a fluorescent material, fluorescent strength emitted from the fluorescent material is measured directly.

More particularly, the kit of the present invention comprises a microtiter plate

adsorbed with an antibody against α 1-acid glycoprotein, haptoglobin or α 2-macroglobulin, or with a lectin, a RCA-horseradish peroxidase complex solution, an ortho-phenylene diamine solution, a phosphate buffer containing Tween as a detergent, a serum dilution, and a standard solution for asialo-glycoprotein.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph illustrating the result on isolation of α 1-acid glycoprotein(AGP) from the blood plasma of a human body by gel filtration using DEAE-cellulose column chromatography.

10 Fig. 2 is a graph illustrating the result on purification of desialylated α 1-acid glycoprotein by gel filtration using Sepadex G-200 column chromatography.

Fig. 3a and Fig. 3b are photographs illustrating results on analysis of purified AGP and desialylated AGP(asialo α 1-acid glycoprotein, AS-AGP) by PAGE and by Western blotting using anti-AGP antibody, respectively, and Fig. 3c and Fig. 3d are the
15 photographs illustrating results on analysis of purified AGP and AS-AGP by SDS-PAGE and by Western blotting using anti-AGP antibody, respectively.

Fig. 4a, Fig. 4b and Fig. 4c are graphs illustrating dose-response depending on the concentration of asialo-glycoprotein by the solid phase sandwich assay using antibody against glycoprotein and lectin. Fig. 4a is of AGP and desialylated AGP, Fig. 4b is of
20 haptoglobin(HG) and desialylated HG, and Fig. 4c is of α 2-macroglobulin(MG) and desialylated MG, respectively.

Fig. 5 is a graph illustrating dose-response depending on the concentration of asialo-glycoprotein i.e., AGP, desialylated AGP, HG, desialylated HG, MG, and desialylated MG by the lectin-lectin solid-phase sandwich assay using a lectin
25 recognizing asialo-glycoprotein, respectively.

Fig. 6a, Fig. 6b and Fig. 6c are graphs illustrating results on measurement of the concentrations of asialo α 1-acid glycoprotein(AS-AGP), asialo haptoglobin(AS-HG), and asialo α 2-macroglobulin(AS-MG) depending on the dilution of serum of a hepatic disease patients by the solid-phase sandwich assay using antibody against glycoprotein and lectin, respectively.

Fig. 7 is a diagram illustrating the measured concentration of the serum AS-AGP of hepatic disease patients by the solid-phase sandwich assay using antibody against AGP and lectin, as an example of measuring the concentrationl of the serum asialo-glycoprotein of hepatic disease patients by sandwich assay using antibody and lectin.

Fig. 8 is a diagram illustrating the measured level of the serum asialo-glycoprotein of hepatic disease patients by the lectin-lectin sandwich assay using lectin recognizing asialo-glycoprotein.

DETAILED DESCRIPTION OF THE EMBODIMENTS

The present invention will be described in more detail with reference to the preferred embodiments. However, the following Examples are only some examples to illustrate the present invention, therefore the scope of the appended claims are not limited to the described Examples.

Example 1: Isolation and Purification of Asialo-glycoprotein from the Serum

α 1-acid glycoprotein(AGP) was isolated and purified from human blood plasma containing glycoprotein by the following method.

To 200mlof human blood plasma was added 2 NIH units of thrombin, allowed to Stand at 37°C for 2 hours and at 4°C overnight, and centrifuged to remove the blood clot. The resulting serum was dialyzed with 0.05M the sodium acetate buffer(pH

4.3), loaded on the DEAE-cellulose column equilibrated with the same buffer, and eluted with a linear concentration gradient from 0.05M to 0.1M sodium acetate buffer(pH 4.3). Thereafter, the absorbance at 280nm of resulting solution was measured and the result is shown in Fig. 1. AGP and fractions containing other proteins were pooled and to them was added 0.5g/ml of ammonium sulfate to precipitate the proteins. After centrifugation, to supernatant was added 0.18g/ml of ammonium sulfate again to precipitate the proteins. This precipitate was dissolved into a small amount of distilled water, dialyzed sufficiently with the distilled water, and lyophilized.

After 40mg of AGP thus isolated was hydrolyzed with 6ml of 0.1N the solution of sulfuric acid solution at 80 °C for 2 hours and neutralized with 1N sodium hydroxide, the resultant was dialyzed with 0.01M phosphate buffer(pH 7.4). 28mg of desialylated α 1-acid glycoprotein(AS-AGP) thus obtained was loaded on the Sepadex G-200 column, gel-filtered with 0.01M phosphate buffer(pH 7.4) to measure its absorbance at 280nm, and pooled fractions containing protein. The result is shown in Fig. 2.

To confirm the characteristics of the isolated AGP and AS-AGP, AGP and AS-AGP were electrophoresed on the polyacrylamide gel and SDS-polyacrylamide gel, dyed with Coomassie blue, and carried out Western blotting analysis for the isolated proteins on gel using anti-AGP antibody(Sigma, USA). The results are shown in Fig. 3a, Fig. 3b, Fig. 3c and Fig. 3d. Fig. 3a and Fig. 3b are photographs illustrating results on analysis of purified AGP and AS-AGP by PAGE and by Western blotting using anti-AGP antibody, respectively. Here, column 1 is that of AGP and column 2 is that of AS-AGP. Fig. 3c and Fig. 3d are photographs illustrating results on analysis of purified AGP and AS-AGP by SDS-PAGE and by Western blotting using anti-AGP antibody, respectively. Here, column 1 is the mark of the standard molecular weight, column 2 is that of AGP, and column 3 is that of AS-AGP.

It can be confirmed that AS-AGP is lower in migrating rate on the polyacrylamide gel than that of AGP because AS-AGP lacking of sialyl group is more positive than AGP, as shown in the result of PAGE of Fig. 3a. As shown the result of SDS-PAGE in Fig. 3c, the molecular weight of AS-AGP due to desialylation is lower than that of AGP. Also, a monoclonal antibody against AGP is shown to react specifically with both of AGP and AS-AGP in the result of Western blotting analysis, as shown in Fig. 3b and Fig. 3d. Therefore, in the present invention, the antibody against glycoprotein was used for measuring serum asialo-glycoprotein.

Example 2: Lectin-Lectin Sandwich Assay Examination

To confirm dose-response depending on the sample concentration by sandwich assay using lectins as both of capture protein and probe protein, the following sandwich assay was carried out by using lectin such as *Arachius hypogaea* agglutinin(PNA), *Ricinus communis* agglutinin(RCA), *Agarius bisporus* agglutinin(ABA), and *Viscum album* agglutinin(VAA) as capture protein and probe protein.

Each 100 μ l of the dilution of 0.13 μ g/ml~4 μ g/ml of the capture lectin was added to each well of a microtiter plate, and allowed to stand overnight at 4 $^{\circ}$ C to adsorb the lectin onto the microtiter plate. Thereafter, 3% the bovine serum albumin solution was added to adsorb the albumin on the remaining spaces of the solid phase surface.

After the wells were washed with the phosphate buffer containing 0.05% Tween(a detergent), each 100 μ l of the double dilution of 0~4 μ g/ml of the AS-AGP obtained in the above example 1 was added to each well to react for 2 hours at room temperature. After washing the wells three times with the above detergent, each 100 μ l of the dilution of 0.05 μ g/ml~0.5 μ g/ml of the probe lectin coupled with horseradish peroxidase(E-Y Laboratories, USA) was added to each well to react for 1 hour at room

temperature. After washing the wells three times with the above detergent, each 100 μ l of the ortho-phenylene diamine(OPD) solution(Sigma, USA) was added to each well to be color-developed. After 15minutes, the reaction was stopped by adding 2.5N sulfuric acid solution and measured its absorbance at 490nm. As a result, dose-response depending on the sample concentration is shown in Table 1.

【Table 1】

Capture Protein	Probe Protein	Dose-response depending on the concentration of AS-AGP in the Sandwich Assay	
		Background	Dose-response
PNA	RCA	Low	High
	VAA	High	Low
	ABA	Low	Low
RCA	PNA	Low	Low
	RCA	Low	High
	VAA	High	Low
	ABA	Low	Low
ABA	PNA	Low	Low
	RCA	Low	Low
	VAA	Low	Low
VAA	PNA	Low	Low
	RCA	Low	Ordinary
	ABA	Low	Low

In the result of Table 1, when PNA or RCA was used as a capture protein, and RCA as a probe protein, dose-response depending on the sample concentration (low background, high dose-response) was satisfactory. A valuation was made as “low” in background, if the OD₄₉₀ value is less than 0.05 and as “high” if more than 0.2, and as “low” in dose-response if the OD₄₉₀ value is less than 0.2, as “ordinary” if 0.2 to 0.8 and as “high” if more than 0.8.

To confirm dose-response depending on the sample concentration by sandwich assay using lectin as a capture protein and antibody as a probe protein, the assay was carried out in the same manner as the Example 2 above described except using an anti-AGP antibody coupled with horseradish peroxidase(E-Y Laboratories, USA) instead of a lectin coupled with horseradish peroxidase as a probe protein. The result is shown in Table 2.

【Table 2】

Capture protein	Probe protein	Dose-response depending on the concentration of AS-AGP in the Sandwich Assay	
		Background	Dose-response
PNA	Anti-AGP antibody	Low	High
RCA		High	High
VAA		Low	Ordinary
ABA		Low	Ordinary

In the result of Table 2, when PNA was used as a capture protein, and anti-AGP antibody as a probe protein, dose-response depending on the sample concentration (low background, high dose-response) was satisfactory. The valuation basis of the background and dose-response is same as the above description for Table 1.

Example 4: Antibody-Lectin Sandwich Assay Examination

By sandwich assay using antibody against AGP, HG or MG as a capture protein and lectin-peroxidase complex as a probe protein, dose-response depending on asialoglycoprotein concentration in the sample was examined as follows.

Each 100 μ l of the appropriately diluted solution of anti-AGP antibody, anti-HG antibody or anti-MG antibody(Sigma, USA) was added to each well of a microtiter plate, and allowed to stand overnight at 4℃ to adsorb the antibody onto the microtiter plate. Thereafter, 3% the bovine serum albumin solution was added to adsorb the

albumin on the remaining spaces of the solid phase surface. After the wells were washed with the phosphate buffer containing 0.05% Tween(a detergent), each 100 μ l of the double dilution of 0.03~4 μ g/ml of the desialylated AGP, HG and MG was added to each well to react for 2 hours at room temperature. After washing the wells three times with the above detergent, each 100 μ l of appropriately diluted solution of the probe lectin coupled with horseradish peroxidase as used in Example 2 was added to each well to react for 1 hour at room temperature. After washing the wells three times with the above detergent, each 100 μ l of the ortho-phenylene diamine solution was added to each well to be color-developed. After 15minutes, the reaction was stopped by adding 2.5N sulfuric acid solution and measured its absorbance at 490nm. As a result, dose-response depending on the sample concentration is shown in Table 3.

【Table 3】

Capture protein	Probe protein	Dose-response depending on the asialo-glycoprotein concentration in the Sandwich Assay	
		Background	Dose-response
Anti-AGP antibody	PNA	High	Ordinary
	RCA	Low	High
	VAA	Low	Low
	ABA	Low	Low
Anti-HG antibody	PNA	Low	Low
	RCA	Low	High
	VAA	Low	Low
	ABA	Low	Low
Anti-MG antibody	PNA	Low	Low
	RCA	Low	High
	VAA	Low	Low
	ABA	Low	Low

In the result of Table 3, when anti-AGP antibody, anti-HG antibody or anti-MG antibody was used as a capture protein, and RCA as a probe protein, dose-response depending on the sample concentration (low background, high dose-response) was

satisfactory. The valuation basis of the background and dose-response is same as the above description for Table 1.

Example 5: Examination of Dose-response depending on the Asialo-

5 glycoprotein Concentration by the Antibody-Lectin Sandwich Assay

To establish a method for measuring asialo-glycoprotein using antibody and lectin, the following sandwich assay was carried out by using a monoclonal antibody adsorbed onto a microtiter plate(solid phase) and a lectin coupled with horseradish peroxidase.

10 That is, each 100 μ l of the appropriately diluted solution of monoclonal antibodies specific for AGP and HG, and polyclonal antibody specific for MG was added to each well of a microtiter plate, and allowed to stand overnight at 4 °C to adsorb the antibody onto the microtiter plate. Thereafter, 3% the bovine serum albumin solution was added to adsorb the albumin on the remaining spaces of the solid phase
15 surface. After the wells were washed with the phosphate buffer containing 0.05% Tween(a detergent), each 100 μ l of the double dilution of 0.03~4 μ g/ml of the AGP, HG, MG, desialylated AGP, desialylated HG and desialylated MG was added to each well to react for 2 hours at room temperature. After washing the wells three times with the above detergent, each 100 μ l of appropriately diluted solution of the lectin-
20 horseradish peroxidase complex was added to each well to react for 1 hour at room temperature. After washing the wells three times with the above detergent, each 100 μ l of the ortho-phenylene diamine solution was added to each well to be color-developed. After 15minutes, the reaction was stopped by adding 2.5N sulfuric acid solution and measured its absorbance at 490nm.

25 As shown in Fig. 4a, Fig. 4b and Fig. 4c, by using the method of the present

invention, desialylated α 1-acid glycoprotein, haptoglobin, and α 2-macroglobulin of the asialo-glycoprotein could be measured in the range of 0.03 μ g/ml~1.0 μ g/ml, respectively.

5 Example 6: Examination of Dose-response depending on the Asialo-glycoprotein Concentration by the Lectin-Lectin Sandwich Assay

Another method for measuring asialo-glycoprotein is sandwich assay using a lectin recognizing asialo-glycoprotein adsorbed onto a solid phase as a capture protein, and a lectin coupled with horseradish peroxidase as a probe protein.

10 That is, each 100 μ l of the dilution of 4 μ g/ml of lectin(RCA, EY Labs.) was added to each well of a microtiter plate, and allowed to stand overnight at 4 $^{\circ}$ C to adsorb the lectin onto the microtiter plate. Thereafter, 1% the bovine serum albumin solution was added to adsorb the albumin on the remaining spaces of the solid phase surface. After the wells were washed with the phosphate buffer containing 0.05% Tween(a
15 detergent), each 100 μ l of the double dilution of 0.03~5.0 μ g/ml of the AGP, HG, MG, desialylated AGP, desialylated HG and desialylated MG was added to each well to react for 2 hours at room temperature. After washing the wells three times with the above detergent, each 100 μ l of appropriately diluted solution of the RCA-horseradish peroxidase complex was added to each well to react for 1 hour at room temperature.
20 After washing the wells three times with the above detergent, each 100 μ l of the ortho-phenylene diamine solution was added to each well to be color-developed. After 15minutes, the reaction was stopped by adding 2.5N sulfuric acid solution and measured its absorbance at 490nm.

As shown in Fig. 5, by using the method of the present invention, desialylated
25 α 1-acid glycoprotein, haptoglobin, and α 2-macroglobulin of the asialo-glycoprotein

could be measured in the range of $0.03\mu\text{ g/ml}$ ~ $5.0\mu\text{ g/ml}$, respectively.

Example 7: Examination of Serum Dilution of Patient by the Anti-Lectin Sandwich Assay Kit

5 A kit for measuring the concentration of asialo-glycoprotein comprising the following components was prepared.

A. solid antibody: an antibody adsorbed onto a microtiter plate. It was prepared by adding each $100\mu\text{ l}$ of an antibody against AGP, HG or MG to each well of the microtiter plate, allowing to stand overnight at 4°C , and then adsorbing an
10 albumin onto the spaces of solid phase surface.

B. lectin coupled with enzyme: RCA solution coupled with horseradish peroxidase(RCA-peroxidase complex)

C. serum dilution

D. ortho-phenylene diamine substrate solution

15 E. detergent: a phosphate buffer containing 0.05% Tween

F. standard solution: a standard solution for asialo-glycoprotein

Using the kit above described, a reaction on dilution of the asialo-glycoprotein in the serum of the healthy subject and patients of liver cirrhosis, hepatocellular carcinoma and chronic hepatitis was examined as follows.

20 The concentration of asialo-glycoprotein was measured by adding each $100\mu\text{ l}$ of the appropriately diluted solution of serum to the A component of solid antibody i.e., an antibody adsorbed onto a microtiter plate well and using components of B, D and E through the sandwich assay as the above described in Example 5.

Fig. 6a, Fig. 6b and Fig. 6c are the serum dilution reaction curves illustrating
25 results obtained by the sandwich kit using anti-AGP antibody and RCA-HRP(in Fig. 6a),

anti-HG antibody and RCA-HRP(in Fig. 6b), and anti-MG antibody and RCA-HRP(in Fig. 6c).

Example 8: Measurement of the Serum Asialo-glycoprotein Concentration of

5 Patients by the Antibody-Lectin Sandwich Kit

The serum asialo-glycoprotein concentrations of patients were measured by the kit used in the Example 7 using an anti-AGP antibody. The concentrations of asialo AGP of the asialo-glycoprotein were measured by the method as described in the Example 5 using dilution in ten times of the serums of the healthy subjects(50 person
10 s), the hepatitis patients(26 persons), liver cirrhosis patients(45 persons), hepatocellular carcinoma patients(37 persons), and non-hepatic disease patients(39 persons). The results showed that, the concentrations of asialo-AGP in liver cirrhosis and hepatocellular carcinoma patients were higher than the normal value statistically, and that of hepatitis patients was similar to the normal value(Fig. 7, Table 4). This means
15 that the increase of the serum asialo-glycoprotein concentration is related to an attack of liver cirrhosis or hepatocellular carcinoma.

Table 4. Serum AS-AGP concentrations as measured by the mAb-RCA sandwich assay

Clinical diagnosis	Total number	AS-AGP > 2.3 ug/mL*		AS-AGP < 2.3 ug/mL*		Mean \pm SD (ug/mL)**
		Number	%	Number	%	
Normal	50	3	6	7	94	1.54 \pm 0.48
Hepatitis	26	1	4	5	96	1.58 \pm 0.46
Liver cirrhosis (LC)	45	42	93		7	3.24 \pm 0.78
Hepatocellular carcinoma (HCC)	20	16	80		20	2.88 \pm 0.73
(LC+HCC)***	17	14	82		18	2.99 \pm 0.73
Non-hepatic disease	39	1	3	8	97	1.08 \pm 0.50

5 * A cut-off value of 2.30ug/mL was used in the analysis.

** Mean \pm SD: Arithmetic mean value of AS-AGP (ug/mL) \pm standard deviation.

*** Patients were diagnosed to have both LC and HCC.

The cut-off value in Fig. 7 was calculated by the sum of the arithmetic mean for
10 serum samples from 50 healthy subjects and double of SD(mean+2SD).

Example 9: Measurement of the Serum Asialo-glycoprotein Concentration of Patients by the Lectin-Lectin Sandwich Kit

A kit for measuring the concentration of asialo-glycoprotein comprising the following components was prepared.

5 A. solid lectin: a lectin adsorbed onto a microtiter plate. It was prepared by adding each 100 μ l of RCA to each well of the microtiter plate, allowing to stand overnight at 4 °C, and then adsorbing 3% bovine serum albumin onto the spaces of solid phase surface.

B. lectin coupled with enzyme: RCA solution coupled with horseradish peroxidase(RCA-peroxidase complex)

C. serum dilution

10 D. ortho-phenylene diamine solution

E. detergent: a phosphate buffer containing 0.05% Tween

The serum asialo-glycoprotein level of patients was measured by the kit above described. The level of asialo AGP of the asialo-glycoprotein was measured by the method as described in the Example 6 using dilution in ten times of the serums of the
15 healthy subjects(41 persons), the hepatitis patients(59 persons), liver cirrhosis patients(98 persons), hepatocellular carcinoma patients(81 persons), and non-hepatic disease patients(53 persons). The results showed that, the serum asialo-glycoprotein concentrations in liver cirrhosis and hepatocellular carcinoma patients were higher than the normal value as that in Example 8. The cut-off value in Fig. 8 was calculated by the
20 sum of the arithmetic mean of absorbance for the serum samples from 41 healthy subjects and double of SD(mean+2SD).

However, the serum asialoglycoprotein concentration of the non-hepatic disease patients was similar to the normal value. Therefore, it shows that the asialo-glycoprotein is specific for the hepatic diseases and can be used as a marker in a diagnosis and
25 judgement of hepatic disease state.

The present sandwich assay method and the kit for measuring serum concentration asialo-glycoprotein can be used effectively in early diagnosis and judgement on treatment result of hepatic diseases including liver cirrhosis and hepatocellular carcinoma, said method being able to measure many samples simultaneously as well as are high in safety and reproducibility.

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WHAT IS CLAIMED IS:

1. A method for measuring asialo-glycoprotein concentration in sample by sandwich assay using lectin as at least one of a capture protein and a probe protein.
 - 5 2. A method according to claim 1 wherein said sample is serum.
 3. A method according to claim 1 which comprises:
 - (a) adsorbing an antibody against glycoprotein onto a solid phase such as a microtiter plate,
 - (b) adding serum sample to the solid phase to bind an asialo-glycoprotein in
10 serum to the antibody,
 - (c) adding a lectin coupled with labeling substance to bind it to the asialo-glycoprotein bound with antibody, and
 - (d) detecting the labeling substance to measure the concentration of asialo-glycoprotein.
 - 15 4. A method according to claim 3 wherein said asialo-glycoprotein is desialylated α 1-acid glycoprotein(AGP), desialylated haptoglobin(HG), desialylated α 2-macroglobulin(MG), or their mixture.
 5. A method according to claim 3 wherein said antibody against glycoprotein is a monoclonal or polyclonal antibody.
 - 20 6. A method according to claim 3 wherein said lectin coupled with labeling substance is *Ricinus communis* agglutinin(RCA) coupled with labeling substance.
 7. A method according to claim 3 wherein said labeling substance is an enzyme or a fluorescent material.
 - 25 8. A method according to claim 3 wherein said asialo-glycoprotein is
-

desialylated α 1-acid glycoprotein(AGP), desialylated haptoglobin(HG), desialylated α 2-macroglobulin(MG), or their mixture, and said lectin is a RCA, and said labeling substance is a horseradish peroxidase.

9. A method according to claim 1 which comprises:

5 (a) adsorbing a lectin recognizing asialo-glycoprotein onto a solid phase such as a microtiter plate,

(b) adding serum sample to the solid phase to bind an asialo-glycoprotein in serum to the lectin,

10 (c) adding a lectin coupled with labeling substance to bind it to the asialo-glycoprotein bound with lectin, and

(d) detecting the labeling substance to measure the concentration of asialo-glycoprotein.

10. A method according to claim 9 wherein said lectin in (a) is a PNA and said lectin coupled with labeling substance in (c) is a RCA coupled with horseradish
15 peroxidase.

11. A method according to claim 9 wherein said lectin in (a) is a RCA and said lectin coupled with labeling substance in (c) is a RCA coupled with horseradish peroxidase.

12. A method according to claim 1 which comprises:

20 (a) adsorbing a lectin recognizing asialo-glycoprotein onto a solid phase such as a microtiter plate,

(b) adding serum sample to the solid phase to bind an asialo-glycoprotein in serum to the lectin,

25 (c) adding an antibody against glycoprotein coupled with labeling substance to bind it to the asialo-glycoprotein bound with lectin, and

(d) detecting the labeling substance to measure the concentration of asialo-glycoprotein.

13. A method according to claim 12 wherein said lectin in (a) is a PNA and said antibody against glycoprotein coupled with labeling substance in (c) is an anti-AGP antibody coupled with horseradish peroxidase.

14. A kit for measuring asialo-glycoprotein concentration by sandwich assay which comprises an antibody against glycoprotein adsorbed onto a solid phase and a lectin coupled with labeling substance.

15. A kit according to claim 14 for measuring asialo-glycoprotein concentration in serum sample.

16. A kit according to claim 14 which comprises an antibody against glycoprotein adsorbed onto a solid phase, a lectin solution coupled with an enzyme, an enzyme substrate solution, a serum sample dilution, a detergent and a standard solution for asialo-glycoprotein.

17. A kit according to claim 16 which comprises an antibody against glycoprotein adsorbed onto a microtiter plate, a horseradish peroxidase-RCA complex solution, an ortho-phenylene diamine solution, a serum sample dilution, a phosphate buffer containing Tween and a standard solution for asialo-glycoprotein.

18. A kit for measuring asialo-glycoprotein concentration in serum sample which comprises an anti-AGP antibody adsorbed onto a microtiter plate, a horseradish peroxidase-RCA complex solution, an ortho-phenylene diamine solution, a serum sample dilution, a phosphate buffer containing Tween and a standard solution for asialo-glycoprotein.

19. A kit for measuring asialo-glycoprotein concentration by sandwich assay which comprises a lectin recognizing asialo-glycoprotein adsorbed onto a solid

phase and a lectin coupled with labeling substance.

20. A kit according to claim 19 for measuring asialo-glycoprotein concentration in serum sample.

21. A kit according to claim 19 which comprises a lectin recognizing
5 asialo-glycoprotein adsorbed onto a solid phase, a lectin solution coupled with an enzyme, an enzyme substrate solution, a serum sample dilution, a detergent and a standard solution for asialo-glycoprotein.

22. A kit according to claim 21 which comprises a lectin recognizing
asialo-glycoprotein adsorbed onto a microtiter plate, a horseradish peroxidase-RCA
10 complex solution, an ortho-phenylene diamine solution, a serum sample dilution, a phosphate buffer containing Tween and a standard solution for asialo-glycoprotein.

23. A kit for measuring asialo-glycoprotein concentration in serum sample
which comprises a RCA adsorbed onto a microtiter plate, a horseradish peroxidase-
RCA complex solution, an ortho-phenylen diamine solution, a serum sample dilution, a
15 phosphate buffer containing Tween and a standard solution for asialo-glycoprotein.

Fig. 1

Isolation of α_1 - acid glycoprotein from human plasma
by DEAE-cellulose column chromatography

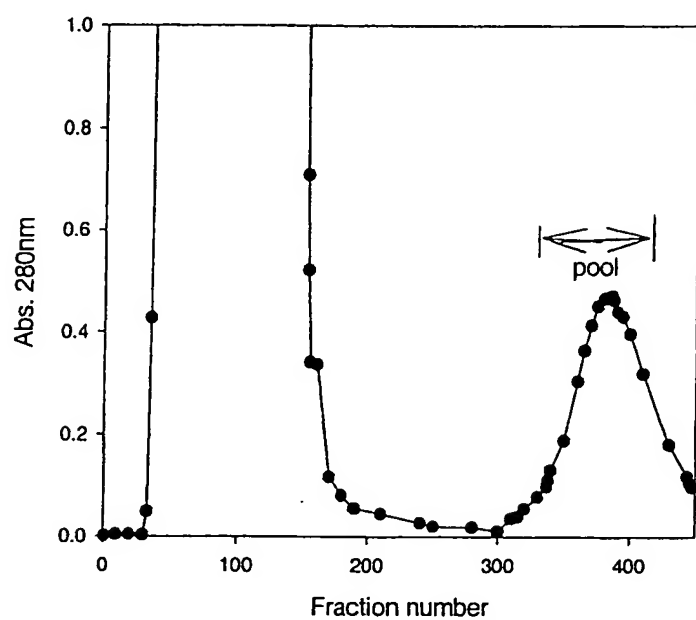


Fig. 2

Purification of α 1-acid glycoprotein by Sephadex G-200 column chromatography

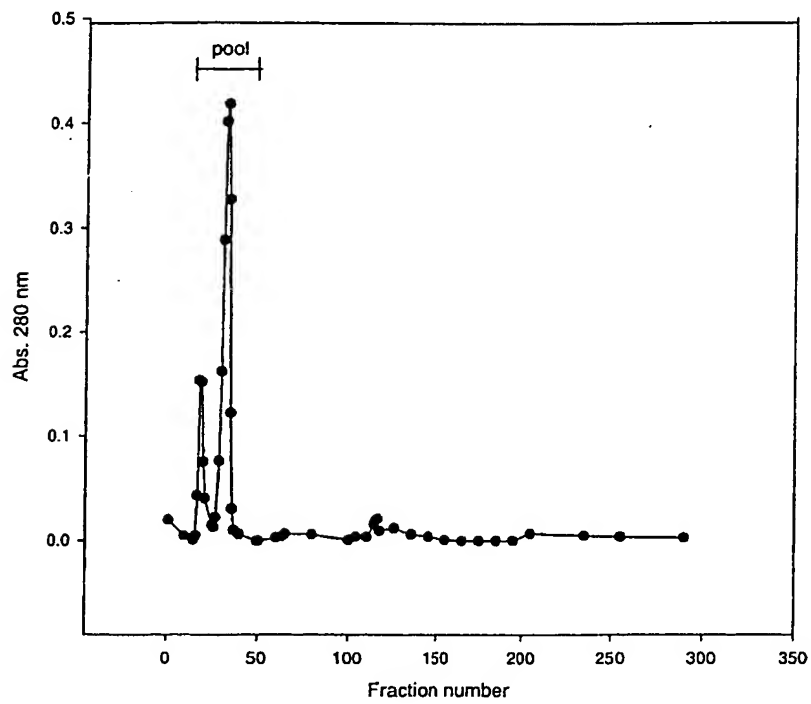
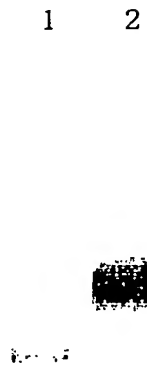


Fig. 3a

Fig. 3b

(A) Polyacrylamide gel electrophoresis

(B) Western blot of (A)



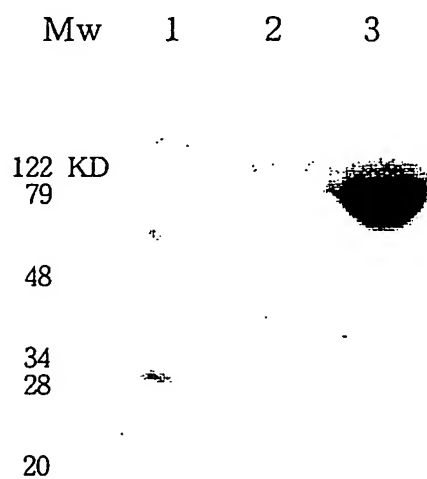
Coomassie
blue stain
blot

Western
using anti-AGP Ab

1. α 1-acid glycoprotein
2. Desialylated α 1-acid glycoprotein

Fig. 3c

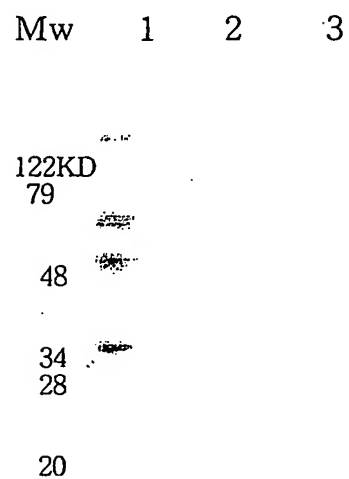
(C) SDS-Polyacrylamide gel electrophoresis



Coomassie blue stain

Fig. 3d

(D) Western blot of (C)

Western blot using
anti-AGP Ab

1. Molecular weight standard markers
2. α 1-acid glycoprotein
3. Desialylated α 1-acid glycoprotein

Fig. 4a

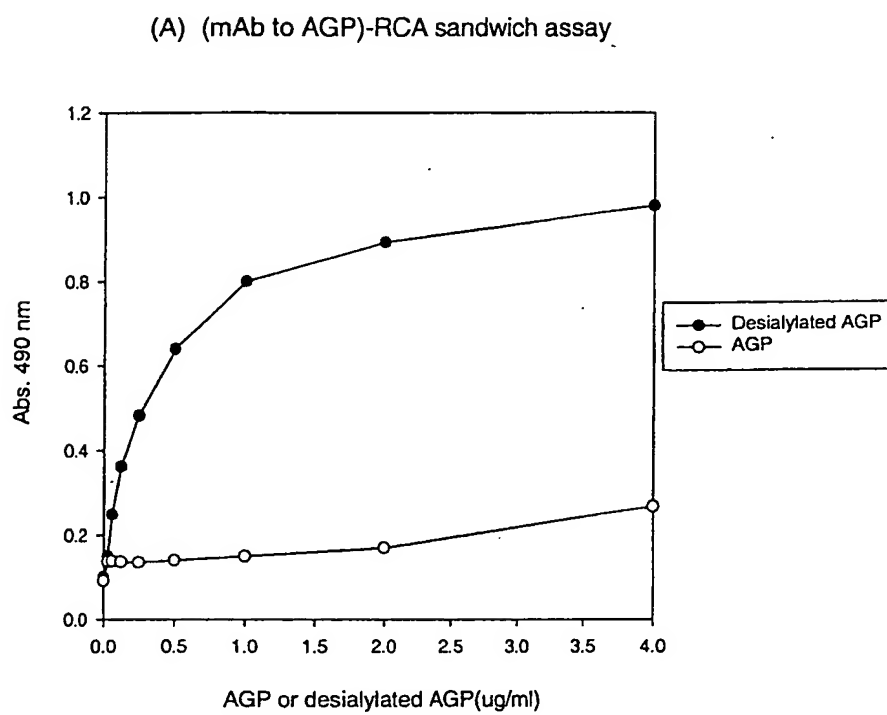


Fig. 4b

(B) (mAb to HG)-RCA sandwich assay

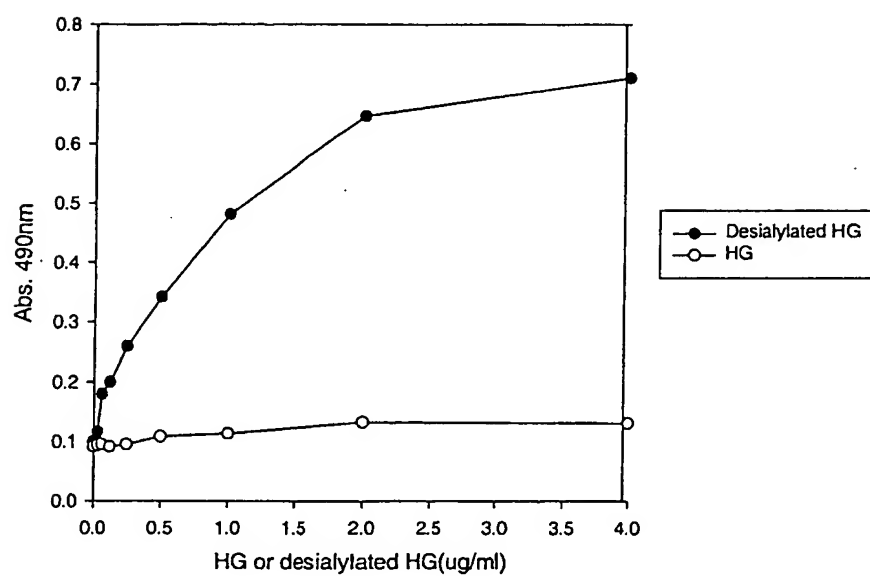


Fig. 4c

(C) (PolyAb to MG)-RCA sandwich assay

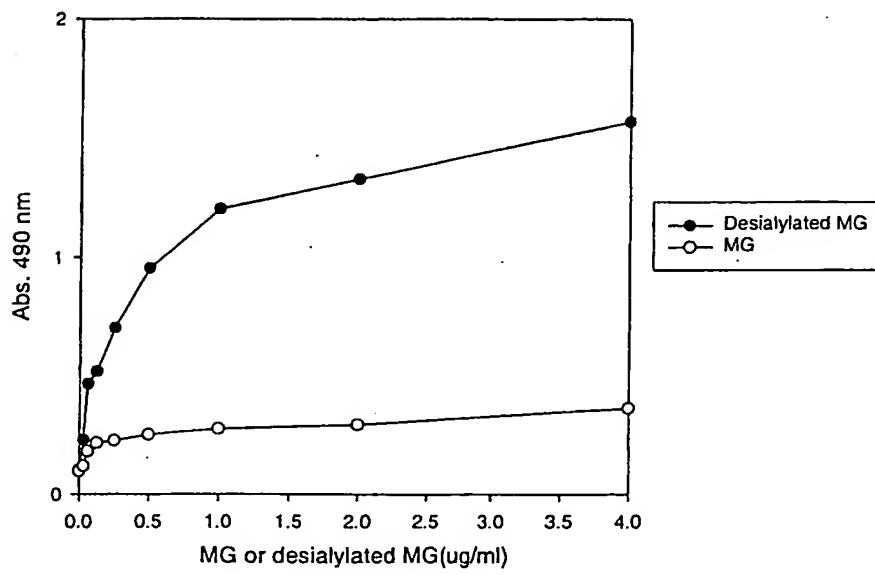


Fig. 5

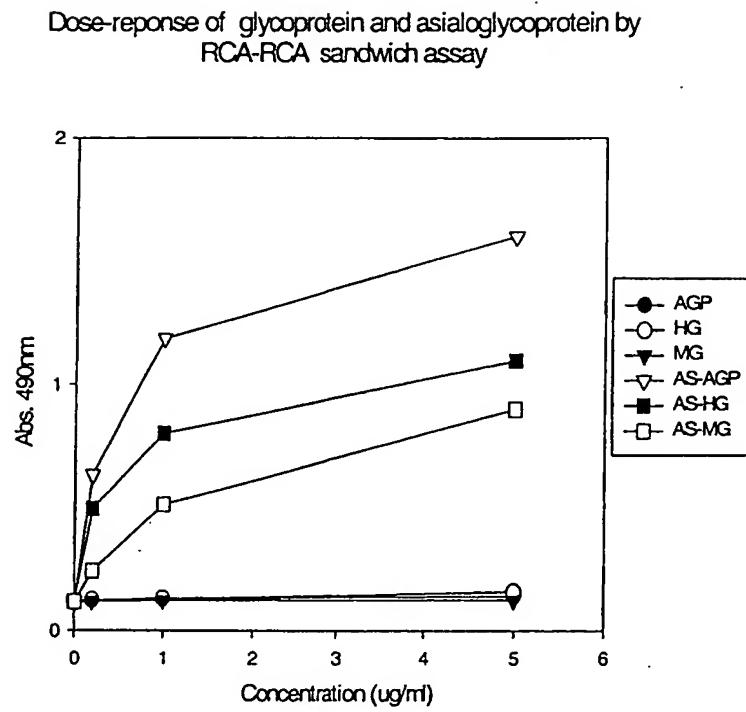


Fig. 6a

(A) (mAb to AGP)-RCA sandwich assay

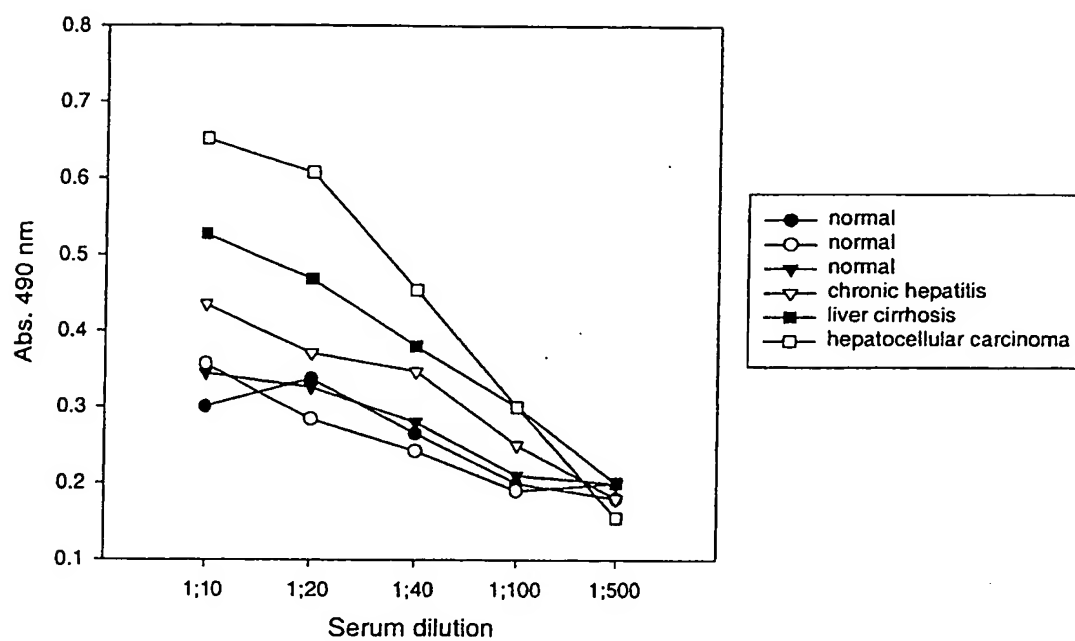


Fig. 6b

(B) (mAb to HG)-RCA sandwich assay

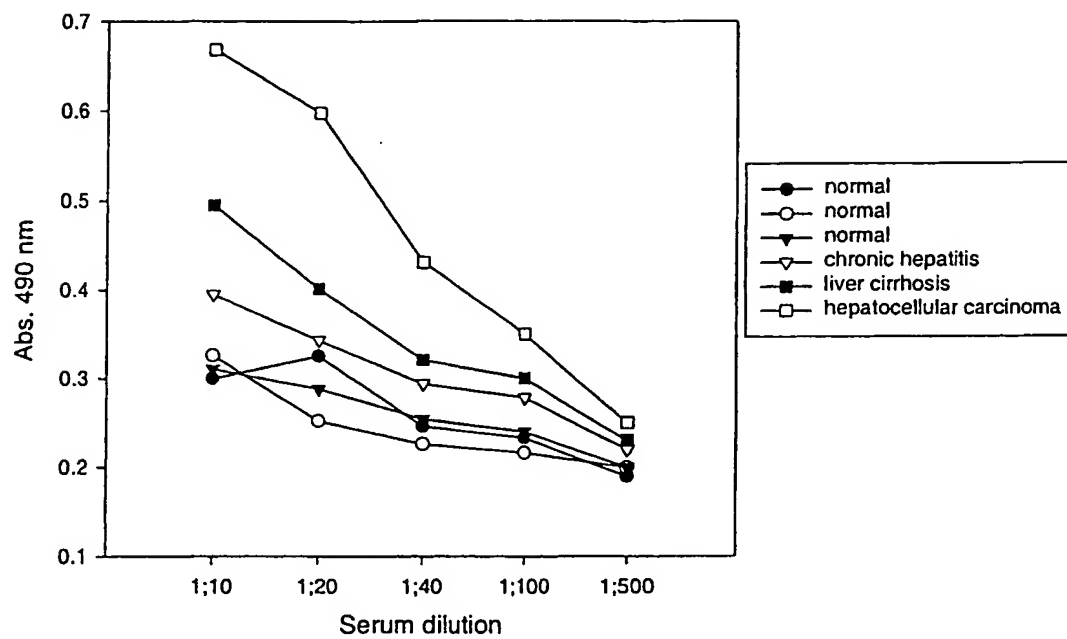


Fig. 6c

(C) (PolyAb to MG)-RCA sandwich assay

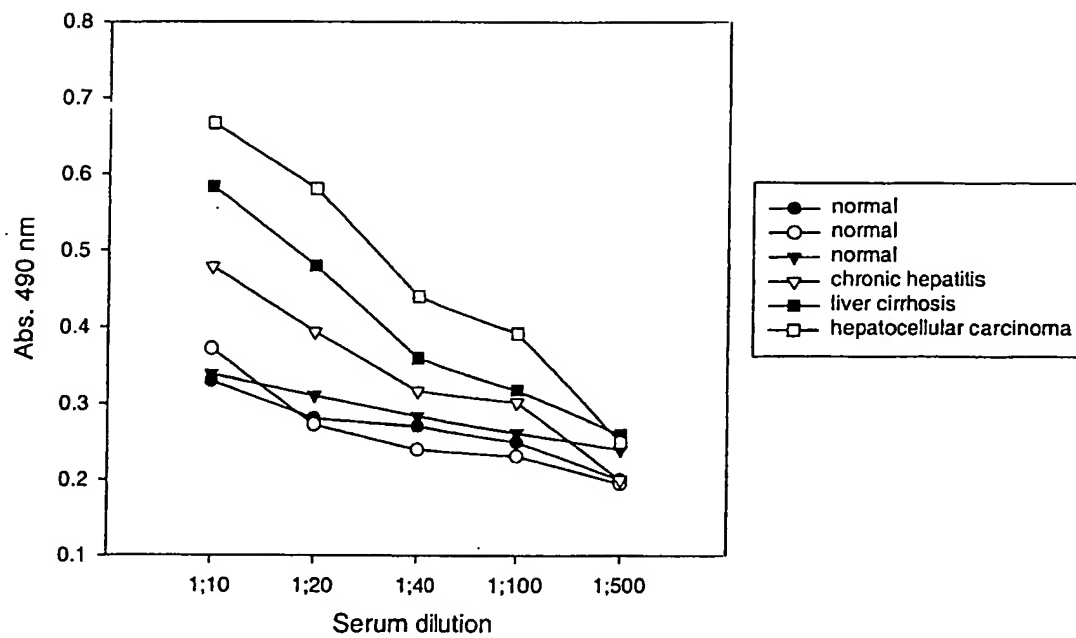
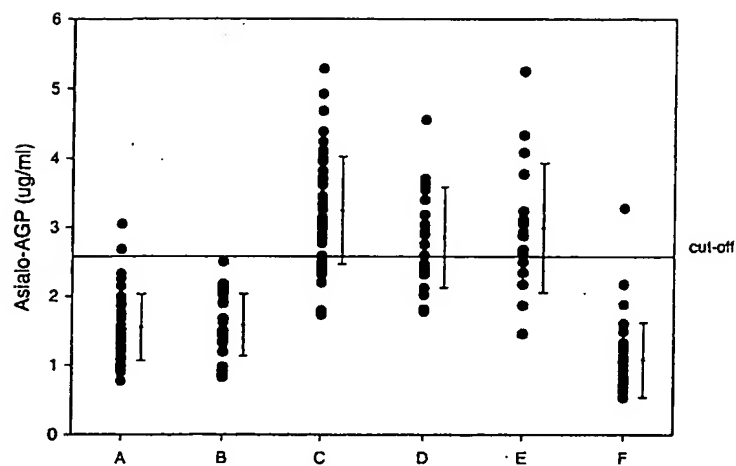
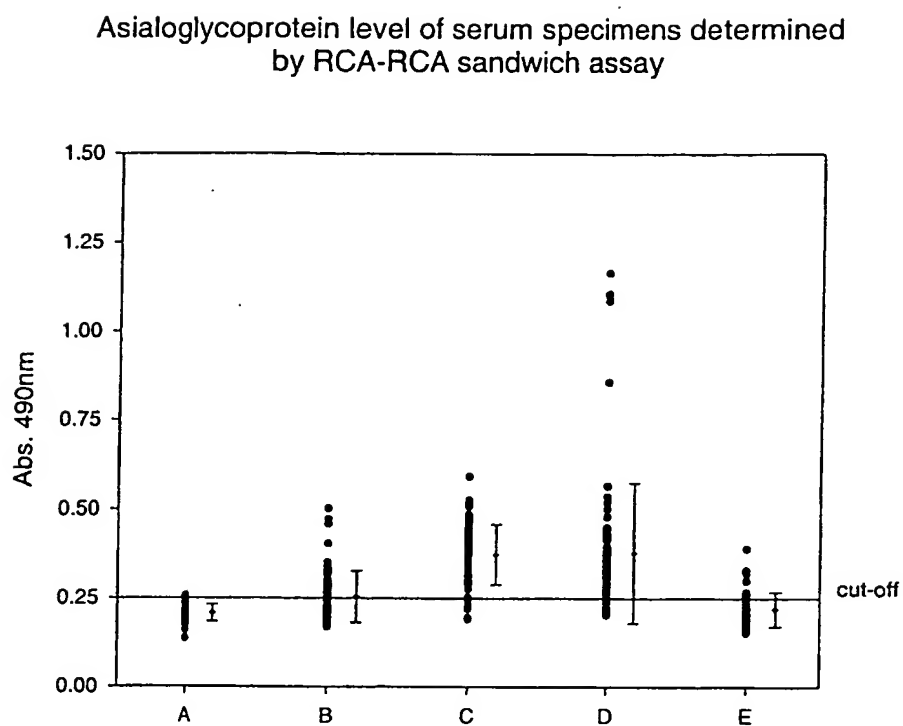


Fig. 7. Serum asialo-AGP levels of normal subjects and patients with liver disease(s)



- A: normal
B: hepatitis
C: liver cirrhosis
D: hepatocellular carcinoma
E: hepatocellular carcinoma+liver cirrhosis
F: non-hepatic diseases

Fig. 8



A: normal

B: hepatitis

C: liver cirrhosis

D: hepatocellular carcinoma

E: non-hepatic disease

The cut-off value represents (arithmetic mean+2SD)
of 41 normal human serum specimens

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 G01N 33/68**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC07 G01N , A61K , A61B, C07K , C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975 ,Korean Utility models and applications for Utility models since 1975,
Japanese Utility models and applications for Utility models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, NPS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUNDY, WISDOM 'An antibody-lectin sandwich assay for quantifying protein glycoforms' In Molecular Biotechnology, Humana Press Inc., 1999 September, Vol. 12, p.203-6 (See introduction and Figure 1)	1,3,6
A	JP 4-356198A (DAICHI PURE CHEM CO. LTD.) 8 DECEMBER 1992 (See the whole document)	1-23
A	JP 9-56380A (TONEN CORP., INTERNATIONAL REAGENTS CORP.) 4 MARCH 1997 (See the whole document)	1-23

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

30 OCTOBER 2000 (30.10.2000)

Date of mailing of the international search report

31 OCTOBER 2000 (31.10.2000)

Name and mailing address of the ISA/KR

Korean Industrial Property Office
Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
Metropolitan City 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer

MIN, Man Ho

Telephone No. 481-5859



Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-13
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1- to 13 are directed a method of diagnostic, the search has been carried out and based on the alleged effects of kit.
2. ☐ Claims Nos.:
because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Search Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be established without effort justifying an additional fee, this Authority did not invite payment of any addition fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR00/00840

Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

JP 4-356198

08,12,1992

NONE

JP 9-56380

04,03,1997

NONE